UKRF 1571 #899

Comm.
Dr. Gardner
Dr. Loosli
Dr. Meier

CHRONIC PULMONARY DISEASE

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

RECEIVED
JAN 30 1973

Date: 1/26/73

1. Principal Investigator (give title and degrees):

Martha F. Greenwood Assistant Professor M. D.

2. Institution & address:

University of Kentucky Medical Center

Lexington, Kentucky 40506

3. Department(s) where research will be done or collaboration provided:

Department of Pediatrics

4. Short title of study:

A Study of the Functional Capacity of the Monocyte-Macrophage System.

5. Proposed starting date: July 1, 1973

6. Estimated time to complete: 3 Years

7. Brief description of specific research aims:

See appended pages

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8. Brief statement of working hypothesis:

See appended pages.

9. Details of experimental design and procedures (append extra pages as necessary)

See appended pages.

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7. Brief Description of Specific Research Aims:

The aim of this project is to investigate in detail the functional capability of cells which collectively form the mononuclear phagocyte system: blood monocyte, alveolar macrophage, macrophages which have undergone in-vitro monocyte to macrophage transformation and the multinucleated giant cell of the respiratory tract.

Specifically, the objectives are:

A) To define the phagocytic and bactericidal capacity of the normal alveolar macrophage.

B) To compare the phagocytic and bactericidal capacity of the blood monocyte, alveolar macrophage and serially throughout the transformation process of the monocyte to macrophage in in-vitro culture.

C) To determine if distinct receptor sites for immunoglobulins, antigen-antibody complexes and complement are present on the plasma membrane of the alveolar macrophage.

D) To prepare an antibody whose specificity is directed toward antigenic sites on the alveolar macrophage surface and to study the effect of this anti-membrane antibody on the attachment and phagocytosis of immunologic and non-immunologic dependent particles by the alveolar macrophage.

E) To study the effect of anti-membrane antibody on cell to cell interlinkage and differentiation of the fibroblast and alveolar macrophage into multi-nucleated giant cells.

F) To quantitatively compare blood monocyte and alveolar macrophage concentrations from animals exposed to tobacco smoke inhalation and controls.

G) To compare the in-vitro cultural characteristics and phagocytic and bactericidal capacity of alveolar macrophages lavaged from animals exposed to tobacco smoke inhalation with normal alveolar macrophage and alveolar macrophages "activated" in-vivo by an agent such as BCG.

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H) To study the effect of smoke exposure on the plasma membrane receptor sites of the alveolar macrophage.

I) To characterize the plasma membrane of the blood monocyte and alveolar macrophage from control and smoked animals and its alteration by anti-membrane antibody using scanning electron microscopy.

J) To develop a method for rapid assay of bactericidal capacity and lysosomal hydrolase content of alveolar macrophages using soft agar plaque technique.

8. Brief Statement of Working Hypothesis:

The importance of the alveolar macrophage, epithelial and connective tissue barriers and the secretory activity of lining cells as pulmonary defense mechanisms is well known. The phagocytic capacity of the alveolar macrophage has been designated as the primary factor in pulmonary clearance of bacteria[1] and inhaled particulates.[2] Our present knowledge concerning the origin of the alveolar macrophage suggests that these cells in the normal steady state are derived from bone marrow precursors, transiently circulate in the blood as mononuclear phagocytes, emigrate through the capillary-alveolar network and subsequently undergo transformation to mature macrophages.[3,4,5,6,7]

Although cigarette smoking has been implicated in a variety of pulmonary disorders the relationship of tobacco smoke to the monocyte-macrophage system is unclear. Previous data on the effect of cigarette smoking on the phagocytic and bactericidal capacity of alveolar macrophages is conflicting.[8,9,10]

An assumption of this proposal is that additional data concerning the basic functional properties of the monocyte-alveolar macrophage system and its response to varied stimuli will allow a clearer understanding of the relationship between these phagocytes and enhanced or impaired pulmonary cellular defense.

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9. Details of Experimental Design and Procedures:

A) Phagocytic and bactericidal assay of normal alveolar macrophages.

Alveolar macrophages will be obtained by lavage of the pulmonary tree from male Macaca speciosa, stump tail monkeys, weighing 2.5-3.5 kgs. using the method of Myrvik, et. al.[11] Animals are sacrificed by intravenous Pentobarbital. Intratracheal pulmonary lavage is performed three times with minimal essential media (MEM) with Heparin, 25 units/ml. The cells are washed X 2 and resuspended in MEM with 10% heat-inactivated fetal calf serum or autologous serum in a final concentration of 2×10^6/ml. Macrophages are then placed in in-vitro culture using 22 mm. glass cover slips in 35 mm. plastic tissue culture dishes or 25 ml. flat bottom tissue culture flasks. Maintenance media is MEM with 20% HIFCS and culture conditions are 37°C in a 5% CO_2-air incubator.

Phagocytic assays are performed using the following test particles: Polystyrene beads, formaldehyde treated erythrocytes, formaldehyde treated bacteria, yeast cell walls (Zymosan), fresh and effete sheep erythrocytes. The percent phagocytosis and phagocytic index will be evaluated using phase microscopy.

Bactericidal capacity will be assayed in alveolar macrophage monolayers grown in flat bottom tissue culture flasks. At the time of assay the culture media is removed and replaced with 10% pooled autologous serum, bacterial suspension and media containing 0.1% gelatin. The bacteria:macrophage ratios to be studied are 1:1 and 1:3. The flasks are incubated on a metabolic shaker at 37°C and at various time periods aliquots are removed and bacterial quantitation of the supernatant fluid is assayed by standard pour plate technique. At the same time intervals, in duplicate flasks, the macrophage monolayer is thoroughly rinsed, adherent macrophages are detached with a rubber policeman, resuspended in dilutions of distilled water for cell lysis and subsequent evaluation of intracellular bacterial survival is performed using pour plate colony

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counts. This method allows evaluation of the rate of phagocytosis of viable organisms as well as the intracellular kill capacity of the macrophage.

B) Comparison of phagocytic and bactericidal capacity of the blood monocyte, alveolar macrophage and during the monocyte to macrophage transformation in-vitro:

Peripheral blood monocytes are isolated using high M.W. Dextran sedimentation and Ficoll-Hypaque gradient centrifugation. The purified population of monocytes obtained are washed and flasks for monocyte bactericidal assay are prepared using the monocyte suspension, bacterial suspension and 20% autologous serum in MEM with 0.1% gelatin. Rotary incubation is carried out at 37°C and aliquots are withdrawn, the monocytes are lysed and bacterial quantitation is performed by standard pour plate techniques following varying intervals of incubation. The phagocytic and bactericidal capacity of the blood monocyte will be determined while in suspension, shortly after attachment to a glass surface and subsequently during the in-vitro transformation of the monocytes to macrophages as previously described.

C) Identification of receptor sites on the alveolar macrophage plasma membrane.

The presence of cell surface immunoglobulin receptor sites will be determined by evaluating the attachment and phagocytosis of opsonized erythrocytes and bacteria by the alveolar macrophage. Antibody coated erythrocytes are prepared using commercial rabbit anti-sheep hemolysin (1/1,500 dilution) by routine technique. This antibody binds to the erythrocyte by its FAB portion and leaves the Fc fragment exposed. Incubation of antibody coated erythrocytes will be used for detecting the presence of "Fc" receptor sites on the alveolar macrophage by observing surface attachment (rosette formation) and subsequent phagocytosis at 37°C. Phagocytosis of opsonized bacteria will be compared with virulent non-opsonized bacterial controls in similar experiments for identification of the

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"Fc" receptor site. The presence of a complement receptor on the alveolar surface will be sought using EAC 1,4,2,3 cells. EAC 1,4,2,3 will be prepared in an adjacent laboratory by Dr. Nancy Holland. EAC 1,4,2,3 will be incubated with monolayers of alveolar macrophages and adherence and rates of phagocytosis determined.

D) Preparation and study of the effect of an anti-membrane antibody on the alveolar macrophage.

Rabbit anti-monkey macrophage antisera will be prepared by injecting rabbits subcutaneously biweekly with a suspension of monkey alveolar macrophages combined with Freund's complete adjuvant. The macrophages are obtained by lavage and cultured in-vitro for 3-4 days. The cover slips are rinsed to remove non-adherent cells and then scraped with a rubber policeman to obtain macrophages which are then combined with Freund's adjuvant. The development in the rabbit of anti-macrophage antibodies is determined by macrophage cytolysis quantitation using fresh quinea pig complement. Since we have previously shown that the macrophage and erythrocyte surface membranes share certain antigenic components,[12] rabbit anti-monkey erythrocyte antisera will also be prepared. Rabbits are injected intraperitoneally with pure populations of washed erythrocytes weekly for 5-6 weeks and the rabbits are bled one week after injection. The anti-macrophage and anti-erythrocyte antisera are heat-inactivated, ammonium sulfate precipitated and purified using Sephadex G-200 columns. The effect of anti-membrane IgG on the primate alveolar macrophage plasma membrane can then be studied.

Phagocytosis by alveolar macrophages following 6-12 hour exposure to non-toxic concentrations of anti-membrane IgG (anti-macrophage and anti-erythrocyte) will be evaluated. Uptake of latex particles, Zymosan, formalinized bacteria and erythrocytes, antibody coated bacteria and erythrocytes and complement coated

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erythrocytes will be compared in antibody exposed alveolar macrophages and controls. These results should allow a further characterization of the alveolar macrophage plasma membrane functional capacity.

E) Long term effect of anti-membrane antibody on cells obtained by pulmonary lavage.

We have previously noted that mouse peritoneal macrophages exposed to anti-membrane antibody exhibit increased cell to cell interlinkage in long term culture. This project proposes to study in detail the effect of anti-macrophage antibody on alveolar macrophages and pulmonary derived fibroblasts. The origin and functional capacity of the multinucleated giant cell is unknown, but fusion of macrophages has been suggested. If anti-membrane antibody alters the alveolar macrophage surface and induces macrophage aggregation, membrane fusion and differentiation into multinucleated giant cells the origin of these cells would be clear and their functional capacity could then be studied.

F,G,H) Study of the functional capacity of tobacco smoke exposed alveolar macrophages.

Using the methods previously described, the in-vitro cultural characteristics, phagocytic function, bactericidal capacity and plasma membrane receptor sites will be evaluated in alveolar macrophages lavaged from animals exposed to tobacco smoke. Smoking procedures will be carried out with the cooperation and consultation of members of the Council for Tobacco Research.

I) Scanning electron microscopy studies.

This project will study the surface membrane of the blood monocyte, normal alveolar macrophage, and alveolar macrophage exposed to smoke and anti-membrane antibody. Procedures for cell preparation are similar to those previously described in SEM studies of the mammalian respiratory tract.[13]

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J) Development of soft agar plaque technique for evaluation of blood monocyte and alveolar macrophage bactericidal capacity.

Agar-gel medium has been previously used for identification of immunoglobulin secreting cells,[14] for the study of blood and bone marrow colony forming units[15] and for quantitative assays of serum and urine lysozyme using Micrococcus lysodeikticus.[16] This project proposes to attempt the development of a similar procedure for evaluation of the monocyte and alveolar macrophages' ability to break down the cell wall of various bacteria.

Blood monocytes or alveolar macrophages are added to warmed agar containing a suspension of bacteria. Aliquots are pipetted into 35 mm. plastic petri dishes and the agar media is allowed to gel at room temperature. The plates are then incubated in a 7.5% CO_2-air mixture in a humidified incubator. Zones of bacterial lysis will be enumerated using a dissecting microscope. If the technique is successful it would allow evaluation following long exposure of phagocytes to bacteria rather than rapid assay as presently required for bactericidal assays. In addition, it would allow multiple bacterial strains to be assayed against a single phagocyte population. Blood monocytes, normal alveolar macrophages, smoke exposed alveolar macrophages and BCG stimulated alveolar macrophages could be compared.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Space and research facilities for this project are available in the Pediatric Hematology research laboratory (MN 469) at the University of Kentucky Medical Center. Facilities and permanent equipment are available in this laboratory for tissue culture, microbial culture, transmission and scanning electron microscopy tissue preparation, phase, fluorescence, cinemicrophotography and routine chemical procedures. Cold room and column preparation space is available in an adjacent area (MN 441). Small animal facilities are also available in an adjacent area (MN 425) and facilities for all animals are available in the general animal quarters. A scanning electron microscope is available for use in the Department of Entomology.

11. Additional facilities required:

None.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

CURRICULUM VITAE

Martha F. Greenwood, M.D.

B.S. Degree:	University of Kentucky, 1964
M.D. Degree:	University of Kentucky College of Medicine, 1968
Internship:	University of Kentucky College of Medicine, 1968-69
Pediatric Residency:	University of Kentucky College of Medicine, 1969-71
Fellowship:	University of Kentucky College of Medicine, 1971-June 30, 1973
Assistant Professor:	University of Kentucky College of Medicine, July 1, 1973

Societies and Honors:

Sophomore Book Award, 1966

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Merck Manual Award, 1968

Outstanding Pediatric Resident Award, 1970-1971

Bibliography:

Greenwood, M.F. and Holland, P.: Scanning electron microscopy of the mammalian respiratory tract. Presented, Society for Pediatric Research, Washington, D. C., April, 1972.

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Greenwood, M.F. and Holland, P.: The mammalian respiratory tract surface. Lab. Invest. 27:296, 1972.

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Holland, P. and Greenwood, M.F.: Mitochondrial iron in sideroblastic anemias. (In Manuscript).

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R: REDACTED MATERIAL

CURRICULUM VITAE

Phillip Holland, M.D.

M.D.:	University of Louisville School of Medicine, Louisville, Kentucky, 1954
Internship:	Cook County Hospital, Chicago, Illinois, 1954-1955.
Service:	R
Pediatric Residency:	The Children's Hospital, Cincinnati, Ohio, 1958-1960
Fellowship:	The Children's Hospital Research Foundation, Cincinnati, Ohio, 1961-1963
Instructor:	University of Cincinnati, College of Medicine, 1962
Senior Research Associate:	The Children's Hospital Research Foundation, Cincinnati, Ohio, 1963-1964
Assistant Professor:	Department of Pediatrics, University of Kentucky School of Medicine, Lexington, Kentucky, 1964-1969
Associate Professor:	Department of Pediatrics, University of Kentucky School of Medicine, Lexington, Kentucky, July 1, 1969-Present
Visiting Associate Professor:	Department of Cellular Immunology, Rockefeller University, New York, New York, July 1, 1970-July 1, 1971

Scientific Societies:

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Abell, C. and Holland, P.: Acute toxoplasmosis complicating leukemia. Am. J. Dis. Child. 118:782, 1969.

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
1. Martha F. Greenwood, M.D.-Principle Investigator	30%	R
2. Phillip Holland, M.D.-Co-investigator	5%	
Technical		
3. Senior Laboratory Technician-to be recruited	100%	7,000.00
Related Fringe Benefits (retirement, life insurance, and Social Security)		1,300.00
	Sub-Total for A	$14,900.00

B. Consumable supplies (by major categories)	
Glassware	500.00
SEM Supplies	300.00
Tissue culture supplies	1,500.00
SEM Rental Time	700.00
Chemicals	500.00
Sub-Total for B	3,500.00

C. Other expenses (itemize)	
Animals-Purchase and Care	1,500.00
Travel	500.00
Printing and Photography	500.00
Sub-Total for C	2,500.00
Running Total of A + B + C	20,900.00

D. Permanent equipment (itemize)

None

Sub-Total for D	0
E. Indirect costs (15% of A+B+C)	3,135.00
Total request	24,035.00

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15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	15,800	3,500	2,500	0	3,270	25,070
Year 3	16,700	3,500	2,500	0	3,405	26,105

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Effect of Tobacco Product Inhalation on the Alveolar Macrophage. (Phillip Holland and Nancy H. Holland)	KTRB 026	44,412	7/1/72-7/1/73

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Continuation of Above	KTRB 026	Same	7/1/73-7/1/74

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Martha F. Greenwood

Signature Martha F. Greenwood Date 1/21/73

Telephone 606 (Area Code) 233-5694 (Number) ____ (Extension)

Checks payable to

University of Kentucky Research Foundation

Mailing address for checks

MN 145, Univ. of Ky. Medical Center

800 Rose Street, Lexington, Kentucky 40506

Responsible officer of institution

Typed Name Carl B. Delabar

Title Associate Director, U.K.R.F.

Signature Carl B. Delabar Date 1-25-73

Telephone 606 (Area Code) 233-5566 (Number) ____ (Extension)

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